

## Molecular and Cytological Studies of an Interspecific Hybrid in *Schoenoplectus* (Cyperaceae)

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ITS sequence and chromosome data were used as evidence of natural hybridization in plants of *Schoenoplectus* (Cyperaceae) from Yawata moor, Hiroshima Prefecture, Japan. The hybrids were intermediate morphologically between both *S. hotarui* and *S. gemmifer*, and *S. hotarui* and *S. triangulatus*, and resembled *S. ×trapezoideus*. Chromosome numbers were observed to be  $2n = 76$  for *S. gemmifer*,  $2n = 44$  for *S. hotarui*,  $2n = 42$  for *S. triangulatus*, and  $2n = 60$  for the putative hybrid. The ITS sequence data showed that the hybrid had sequences from both *S. gemmifer* ( $2n = 76$ ) and *S. hotarui* ( $2n = 44$ ). Our cytological and ITS sequence results suggested that the putative hybrid with  $2n = 60$  arose by natural hybridization between *S. gemmifer* ( $2n = 76$ ) and *S. hotarui* ( $2n = 44$ ).

Keywords: Chromosome number, Cyperaceae, hybrid, ITS sequence, *Schoenoplectus*

The genus *Schoenoplectus* (Rchb.) Palla (Cyperaceae), including about 77 species, is distributed worldwide, mainly in marshes and other wetland habitats (Hayasaka 2002). Within the genus, many natural interspecific hybrids have been reported by numerous authors (Koyama 1958, Smith 1969, Hayasaka 2002, Hayasaka & Ohashi 2000, Yashiro 2004, Hayasaka 2005). Hayasaka (2002) recognized 22 hybrids in his monograph of *Schoenoplectus*, 12 hybrids of which are members of section *Actaeogeton* (Rchb.) J. Raynal. Most of these reports were inferred to be natural interspecific hybrids based on intermediate morphological characters between their putative parents and were growing sympatrically with their putative parents.

Cytological or molecular studies of natural interspecific hybrids and their parental species in *Schoenoplectus* have been reported by Otzen (1962), Greef & Triest (1999), Fay *et al.* (2003),

and Yano & Hoshino (2005). Otzen (1962) reported chromosome numbers of  $2n = 42$  for three hybrids, between *S. tabernaemontani* (C. C. Gmel.) Palla and *S. triqueter* (L.) Palla, between *S. lacustris* (L.) Palla and *S. tabernaemontani*, between *S. lacustris* and *S. triqueter*. Greef & Triest (1999) and Fay *et al.* (2003) reported evidence of natural interspecific hybrid between *S. tabernaemontani* and *S. triqueter*, based on random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) analysis. Most of these works, however, were conducted on hybrids involving *S. tabernaemontani*, *S. lacustris*, and *S. triqueter* within section *Schoenoplectus*. Yano & Hoshino (2005) reported that two hybrids, *S. ×trapezoideus* (Koidz.) Hayas. & H. Ohashi and *S. ×uzenensis* (T. Koyama) Hayas. & H. Ohashi, had an intermediate chromosome number between both putative parents in section *Actaeogeton*, respectively. Hayasaka (2002) re-

ported that intersectional hybrids are not known in *Schoenoplectus*. To clarify the parentage of natural hybrids in the genus, more cytological and molecular studies of hybrids within section *Actaeogeton*, including many natural interspecific hybrids (Hayasaka 2002), are necessary.

Recently, nuclear ribosomal internal transcribed spacer (ITS) sequences have been commonly used in phylogenetic analyses and have proven useful in lower-level relationships in Cyperaceae (Starr *et al.* 1999, Hendrichs *et al.* 2004a, b, Roalson & Friar 2000, 2004, Yano *et al.* 2004, Yano & Hoshino 2005, 2006). With ITS sequence data it is necessary to consider the presence of ITS intraspecific polymorphisms, paralogous copies of ITS in the genome, and pseudogene copies of the nrDNA repeat (Roalson & Friar 2004). Yano & Hoshino (2005) reported that polymorphic sequences derived from paralogous or pseudogene copies of the nrDNA were not found in the ITS region of the genus *Schoenoplectus*, but hybrids of *Schoenoplectus* were not included in their study. In the hybrids, the ITS sequences often show genic recombination of the sequences from the parental species or a polymorphic pattern indicative of the presence of both parental repeat types (Alvarez & Wendel 2003). Analyses using ITS sequences provide important evidence for clarifying natural hybridization in *Schoenoplectus*.

We found putative hybrids of *Schoenoplectus* from Yawata moor in the northwestern part of Hiroshima Prefecture in western Honshu, Japan. Yawata moor is situated on the Yawata highland, about 800 m above sea level (Horikawa *et al.* 1959), where *S. gemmifer* C. Sato, T. Maeda & Uchino, *S. hotarui* (Ohwi) Holub, *S. nipponicus* (Makino) Soják, *S. tabernaemontani*, *S. triangulatus* (Roxb.) Soják, and *S. triqueter* were found. The putative hybrid was morphologically intermediate between both *S. hotarui* and *S. gemmifer*, and *S. hotarui* and *S. triangulatus*, and resembled *S. ×trapezoideus*.

The goal of the present study was to clarify the hybrid origin of the putative hybrid from Yawata moor using chromosome and ITS sequences data and to discuss the relationship be-

tween the putative hybrid and *Schoenoplectus ×trapezoideus* and the parental species.

## Materials and Methods

Karyomorphological observations were conducted on two individuals of the putative hybrid, five individuals of *Schoenoplectus hotarui*, two individuals of *S. gemmifer*, and three individuals of *S. triangulatus* from Yawata moor. ITS sequences of the putative hybrids and allied species were determined for the one individual of the putative hybrid from Yawata moor, one individual of *S. gemmifer*, two individuals of *S. triangulatus*, and two individuals of *S. ×trapezoideus*. We also used our previously published ITS data of section *Actaeogeton* in *Schoenoplectus* (Yano & Hoshino 2005). Voucher specimens and their localities are listed in Table 1. Voucher specimens are deposited in the herbaria of Okayama University of Science (OKAY) and Tohoku University (TUS).

Somatic chromosomes were observed in the meristematic cells of root tips. Root tips were pre-treated in 2 mM 8-hydroxyquinoline solution for 1 h at 23°C and then 15 h at 4°C. They were fixed in acetic alcohol (1:3) for more than 16 h at -20°C or for 1.5 h. The fixed roots were hydrated in an alcohol series (70, 30, 15%, respectively) and distilled water, then hydrolyzed with 1N HCl for 10 min at 60°C, and stained with leuco-basic fuchsin for 1 h at 23°C. The samples were then macerated in a mixture of 2% pectinase and 2% cellulase for 1 h at 37°C, restained in 1% aceto-orcein, and squashed.

Total DNA was extracted from 0.1 g fresh material using a Nucleon Phytopure plant and fungal DNA extraction kit (Amersham Inc.). Amplification of the nrITS region was performed by polymerase chain reaction (PCR) with Taq polymerase (TaKaRa Inc.). Reactions were performed using a DNA thermal cycler (Program Temp Control System PC-320, Astec Inc.) for the ITS region, following the protocol of Hsiao *et al.* (1994). The entire ITS-region was amplified with primers ITS1 (Hsiao *et al.* 1994) and ITS4 (White *et al.* 1990). PCR products were electrophoresed

TABLE 1. Locality, voucher, and chromosome numbers of putative hybrids and parental species used in this study. Genbank accession numbers listed for ITS data. Vouchers are deposited in OKAY and TUS. Asterisk indicates chromosome numbers from previous report by Yano & Hoshino (2005).

Taxon	Chromosome number (2n)	Accession No. ITS
<i>Schoenoplectus gemmifer</i> C. Sato & al.		
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19678 (OKAY)	76	
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19685 (OKAY)	76	AB523848
<i>S. hotarui</i> (Ohwi) Holub		
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19677-1, 19677-2 (OKAY)	44	
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19682-1, 19682-3, 19682-4 (OKAY)	44	
<i>S. triangulatus</i> (Roxb.) Soják		
Japan, Shizuoka Pref., Hamamatsu-shi, Higashi-kandagawa; Kitamura 19556 (OKAY)	42	AB523849
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19680-2 (OKAY)	42	AB523850
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19681-1, 19681-2 (OKAY)	42	
<i>S. ×trapezoideus</i> (Koidz.) Hayas. & H. Ohashi		
Japan, Miyagi Pref., Sendai-shi, Saikachi; Hayasaka 2483 (TUS)	43*	AB523851
Japan, Tochigi Pref., Utsunomiya-shi, Tsuruta; Noguchi 20489 (OKAY)		AB523852
hybrid		
Japan, Hiroshima Pref., Yamagata-gun, Yawata; Hoshino & al. 19679 (OKAY)	60	AB523853

on 1.4% agarose gels to confirm a single product, and purified using a Qiaquick PCR purification Kit (Qiagen Inc.). Cycle sequencing reactions were performed using the purified PCR products and a BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems Inc.). For ITS sequencing, one of the amplification primers, ITS1 and ITS4, or one of the internal sequencing primers ITS2C (Yano *et al.* 2004) and ITS3C (Yano *et al.* 2004) were used. The products were resolved by capillary electrophoresis using an ABI PRISM 310 automated DNA sequencer. Sequences were assembled and edited using Sequencing Analysis version 3.0 (Perkin Elmer Inc.). The data matrices of the ITS sequences of the species of *Schoenoplectus* were aligned using Clustal W (Thompson *et al.* 1994) and then adjusted manually as necessary.

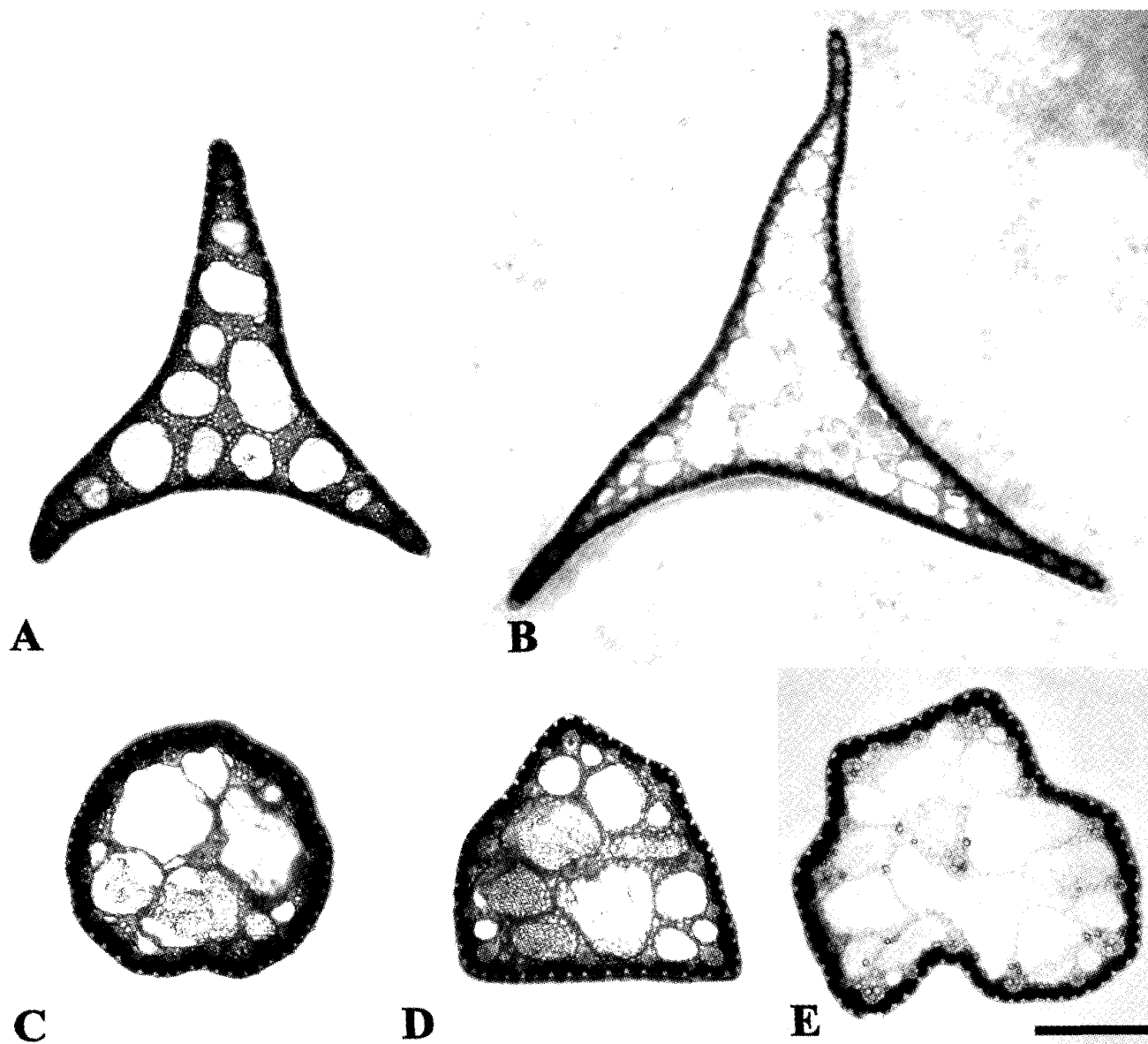
## Results and Discussion

### Morphological characters

The putative hybrid from Yawata moor showed intermediate morphological features between *Schoenoplectus gemmifer* and *S. hotarui*, or between *S. triangulatus* and *S. hotarui* (Table 2). The intermediate morphological characters mainly in the shape of the culms (Fig. 1). The culms of *S. gemmifer* and *S. triangulatus* are acutely three angled, but the culm of *S. hotarui* is cylindrical. The culms of the putative hybrid from Yawata moor are three angled (Fig. 1). Although the *S. gemmifer* and *S. triangulatus* are closely related, *S. gemmifer* differs from *S. triangulatus* in having two stigmas, linear leaf blades, and gemmae (Sato *et al.* 2004). *Schoenoplectus hotarui* has three stigmas, no gemmae, and bladeless leaves (Table 2). The putative hybrid from Yawata moor has two or three stigmas, gemmae are present or absent, and the leaves are bladeless

TABLE 2. Morphological comparison of putative hybrids and parental species of *Schoenoplectus*.

	<i>S. gemmifer</i>	hybrid	<i>S. hotarui</i>	<i>S. juncooides</i>	<i>S. ×trapezoideus</i>	<i>S. triangulatus</i>
Culm shape	acutely 3-angled	3-angled	cylindrical	cylindrical-several or angled	3–5-angled	acutely 3-angled
Culm diameter	2–5 mm	1–1.5 mm	1–2 mm	1–3 mm	1–3 mm	3–10 mm
Leaf blade	linear or bladeless	bladeless	bladeless	bladeless	bladeless	bladeless
Spikelet form	ovoid or oblong-ovoid, apex subacute	ovoid or oblong-ovoid, apex subacute	ovoid, apex rounded	ovoid or oblong-ovoid, apex acute	ovoid or oblong-ovoid, apex subacute	oblong-ovoid, apex acute
Stigma	2	2 or 3	3	2	3	3
Gemma	present or absent	present or absent	absent	absent	absent	absent

FIG. 1. Transverse sections of culms. A: *Schoenoplectus gemmifer*. B: *S. triangulatus*. C: *S. hotarui*. D: hybrid. E: *S. ×trapezoideus*. Bar = 0.5 mm.

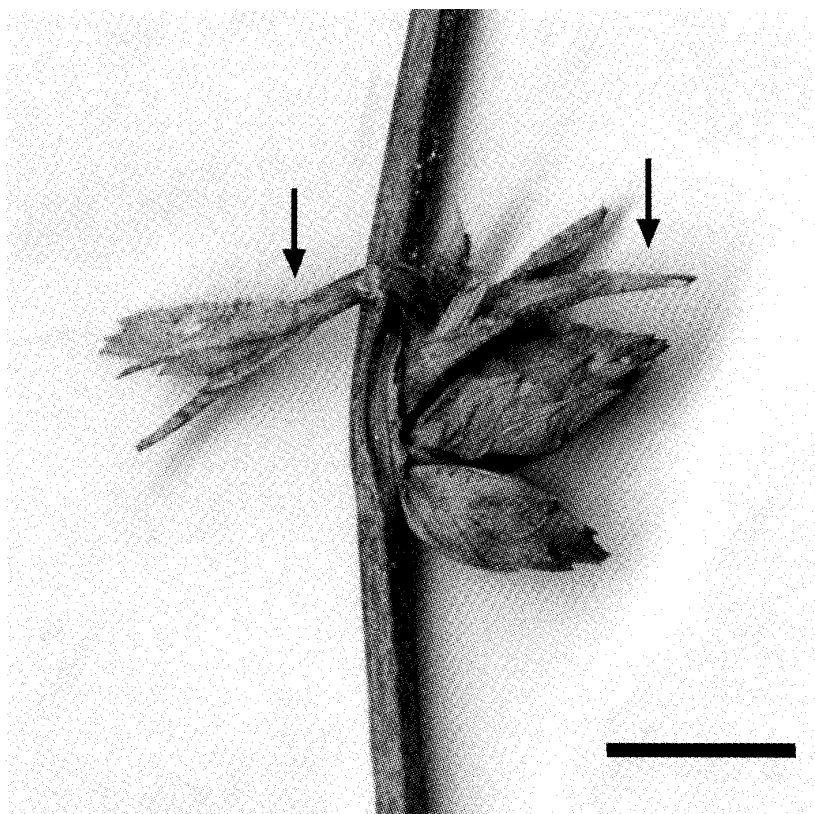


FIG. 2. Inflorescence of putative hybrid from Yawata moor. Arrows indicate gemmae. Bar = 5 mm.

(Table 2). Linear leaf blades and gemmae are diagnostic characters of *S. gemmifer* of section *Actaeogeton* of *Schoenoplectus*. The putative hybrid had gemma, and was thought to be closely related to *S. gemmifer* (Fig. 2). The results suggest that the hybrid, with its intermediate features, is a putative hybrid between *S. gemmifer* and *S. hotarui*.

Some individuals of the putative hybrid in Yawata moor, however, had three stigmas, no gemmae, and were very similar to *Schoenoplectus*  $\times$  *trapezoideus* (Table 2). *Schoenoplectus*  $\times$  *trapezoideus* is considered to be a natural hybrid between *S. hotarui* and *S. triangulatus*, or between *Scirpus juncoides* Roxb. var. *ohwianus* T. Koyama and *S. triangulatus*, based on morphological data (Koyama 1958, Hayasaka 2002). *Schoenoplectus*  $\times$  *trapezoideus* also shows intermediacy in the shape of culm between the putative parental species (Fig. 1). The evidence indicates that discriminating the two putative hybrids is difficult, because the morphological characters

of these hybrids and their putative parental species overlap.

#### Chromosome observations

The chromosome numbers determined in this study are shown in Table 1. The putative hybrid from Yawata moor had a chromosome number of  $2n = 60$  (Fig. 3A). Chromosome numbers were observed to be  $2n = 76$  for *Schoenoplectus gemmifer* (Fig. 3B),  $2n = 44$  for *S. hotarui* (Fig. 3C), and  $2n = 42$  for *S. triangulatus* (Fig. 3D), all from Yawata moor, which confirm previous reports (Yano & Hoshino 2005). Somatic chromosomes of these species were small, and ranged gradually from 0.5 to 1.3  $\mu\text{m}$  in length.

Koyama (1958) suggested that *Schoenoplectus*  $\times$  *trapezoideus* is a hybrid between *Scirpus juncoides* var. *ohwianus* and *S. triangulatus*, while Hayasaka (2002) reported that *S.*  $\times$  *trapezoideus* is considered to be a natural hybrid between *S. hotarui* and *S. triangulatus*. Yano &

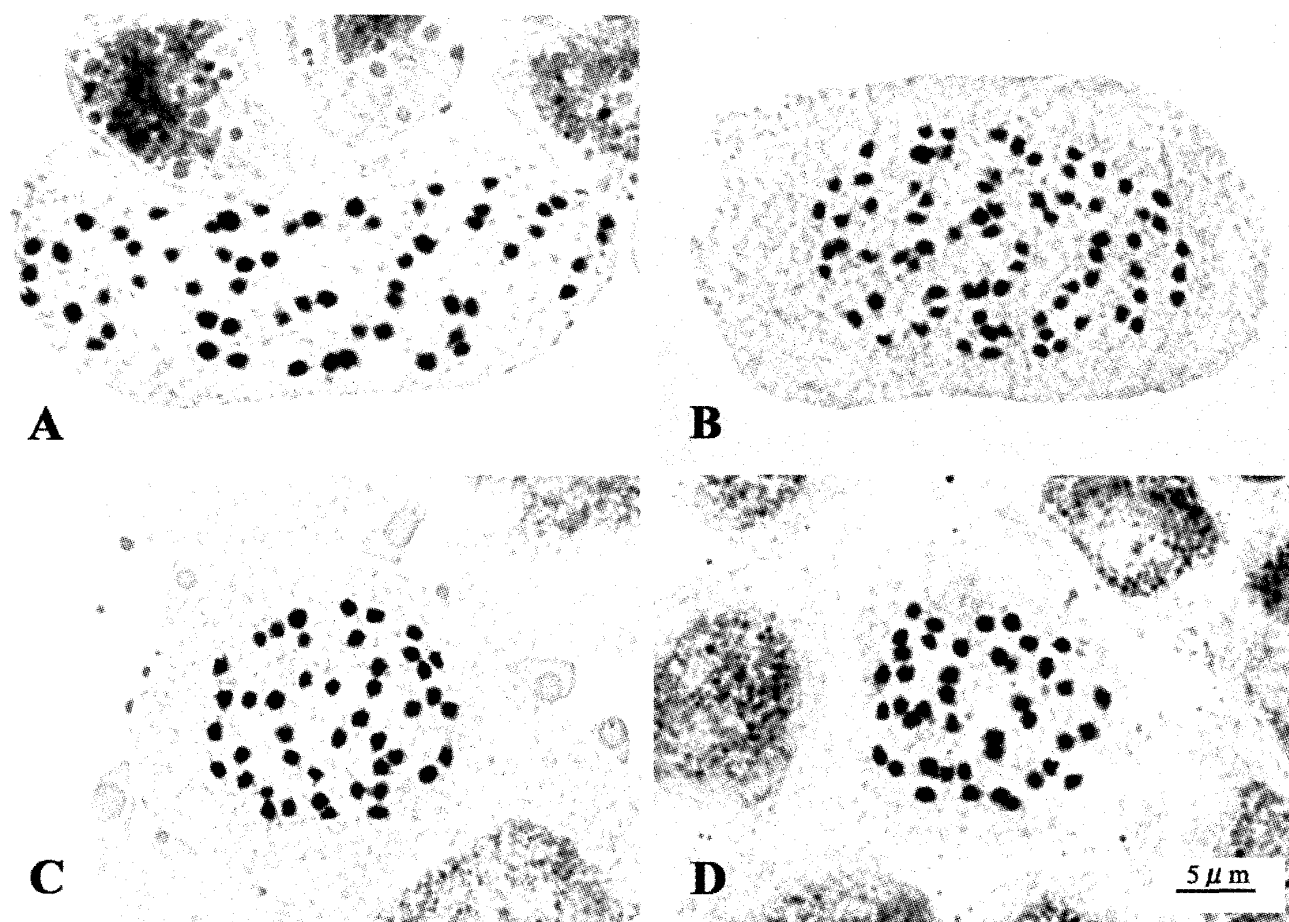


FIG. 3. Photomicrographs of somatic metaphase chromosomes of putative hybrid and parental species of *Schoenoplectus*. A: hybrid ( $2n = 60$ ). B: *S. gemmifer* ( $2n = 76$ ). C: *S. hotarui* ( $2n = 44$ ). D: *S. triangulatus* ( $2n = 42$ ).

Hoshino (2005) reported  $2n = 43$  for *S. ×trapezoideus*,  $2n = 42$  and  $44$  for *S. hotarui*,  $2n = 42$  for *S. triangulatus*, and  $2n = 74$  for *S. juncoides*. They also suggested that *S. ×trapezoideus* arose from natural hybridization or introgression between *S. hotarui* ( $2n = 44$ ) and *S. triangulatus* ( $2n = 42$ ), because *S. ×trapezoideus* has a chromosome number intermediate between the both putative parents (i.e.,  $2n = 43 = 22 + 21$ ). In the present study, the putative hybrid from Yawata moor showed  $2n = 60$  and might have originated from hybridization between plants with  $2n = 76$  and  $2n = 44$  (i.e.,  $2n = 60 = 38 + 22$ ). Thus, the putative hybrid in Yawata moor is thought to be a natural hybrid between *S. gemmifer* and *S. hotarui*, and not *S. ×trapezoideus*.

#### Comparison of nrITS sequences

The complete ITS (ITS1–5.8S–ITS2) sequences of two putative hybrids and their putative parental species were 621 bp in length. Aligned sequences of the 13 species of section *Actaeogeton* in *Schoenoplectus*, including two putative hybrids were 625 bp. The numbers of polymorphic characters were 46 nucleotide substitutions and four indels in the ITS region (Table 3). Specific diagnostic sites in the ITS sequence were found among 13 species of section *Actaeogeton* (Table 3). The ITS sequences of *S. gemmifer* ( $2n = 76$ ) and *S. hotarui* ( $2n = 44$ ) were different at seven nucleotide sites. The putative hybrid from Yawata ( $2n = 60$ ) showed additive nucleotide sites, G/A or T/C, of both *S. gemmifer* ( $2n = 76$ ) and *S. hotarui* ( $2n = 44$ ) at all of the nucleotide substitution sites

TABLE 3. Variable sites of nrITS sequences in *Schoenoplectus* section *Actaeogeton*. Number after each taxon is chromosome number (2n). Asterisk indicates chromosome number from previous reports by Iwasaki & Ueki (1979) and Yano & Hoshino (2005).

Taxon (2n)	Accession no.	ITS1–5.8S–ITS2 (Locus site; bp)																	
		2	8	9	10	11	27	28	32	35	51	52	72	74	86	87	95	100	
<i>S. komarovii</i> (38*)	AB206261	T	C	T	C	T	C	A	G	C	T	C	G	C	C	C	C	C	
<i>S. hondoensis</i> (38*)	AB206258	T	C	T	C	T	C	A	A	G	C	T	C	G	C	C	T	C	
<i>S. hotarui</i> (42*)	AB180720	C	T	C	T	T	C	A	A	G	C	T	C	G	C	C	T	C	
<i>S. hotarui</i> (44*)	AB206259	T	C	T	C	T	C	A	A	G	C	T	C	G	C	C	T	C	
<i>S. mucronatus</i> (38*)	AB206263	T	C	T	C	T	C	A	A	G	C	C	C	G	C	C	T	C	
<i>S. triangulatus</i> (42)	AB206265	T	C	T	C	C	C	A	A	G	T	C	C	G	C	C	T	C	
	AB523849	T	C	T	C	C	C	A	A	G	T	C	C	G	C	C	T	C	
	AB523850	T	C	T	C	C	C	A	A	G	T	C	C	G	C	C	T	C	
	AB523851	T	C	T	C	C/T	C	A	A	G	C/T	C/T	C	G	C	C	T	C	
<i>S. ×trapezoideus</i> (43*)	AB523852	T	C	T	C	C/T	C	A	A	G	C/T	C/T	C	G	C	C	T	C	
	AB523853	T	C	T	C	T	C	A	A	G	C	C/T	C	A/G	C	C	T	C	
<i>S. gemmifer</i> (76)	AB523848	T	C	T	C	T	C	A	A	G	C	C	C	A	C	C	T	C	
	AB206257	T	C	T	C	T	C	A	A	G	C	C	C	A	C	C	T	C	
<i>S. multisetus</i> (70*)	AB206264	T	C	T	C	T	C	A	A	G	C	C	C	A	C	C	T	C	
<i>S. wallichii</i> (72*)	AB206266	T	C	T	C	T	C	A	A	C	T	C	C	G	T	C	T	C	
<i>S. juncoides</i> (74*)	AB206260	T	C	T	C	T	C	G	A	C	T	C	C	G	T	C	T	C	
<i>S. lineolatus</i> (74*)	AB206262	T	C	T	C	T	T	A	A	C	T	C	T	G	C	T	T	T	

		144	157	177	185	200	211	385	389	394	395	417	423	442	443	459	477	484
<i>S. komarovii</i> (38*)	AB206261	C	C	C	C	C	C	G	C	C	C	G	C	C	C	G	C	C
<i>S. hondoensis</i> (38*)	AB206258	C	C	C	C	C	C	G	C	C	C	G	C	T	C	G	C	C
<i>S. hotarui</i> (42*)	AB180720	C	C	C	C	C	C	G	C	C	C	G	C	T	C	G	C	C
<i>S. hotarui</i> (44*)	AB206259	C	C	C	C	T	C	G	C	C	C	G	C	T	C	G	C	C
<i>S. mucronatus</i> (38*)	AB206263	C	C	C	C	C	C	A	C	C	C	G	C	C	C	G	C	C
<i>S. triangulatus</i> (42)	AB206265	C	C	C	C	C	T	G	C	C	T	G	C	C	C	G	C	C
	AB523849	C	C	C	C	C	C	G	C	C	T	G	C	C	C	G	C	C
	AB523850	C	C	C	C	C	C/T	G	C	C	T	G	C	C	C	G	C	C
<i>S. ×trapezoideus</i> (43*)	AB523851	C	C	C	C	C	C	G	C	C	C/T	G	C	C/T	C	G	C	C
	AB523852	C	C	C	C	C	C	G	C	C	C/T	G	C	C/T	C	G	C	C
hybrid (60)	AB523853	C	C	C	C	C	C	G	C	C	C	A/G	C	C/T	C	G	C	C
<i>S. gemmifer</i> (76)	AB523848	C	C	C	C	C	C	G	C	C	C	A	C	C	C	G	C	C
	AB206257	C	C	C	C	C	C	G	C	C	C	A	C	C	C	G	C	T
<i>S. multisetus</i> (70*)	AB206264	C	C	C	C	C	C	G	C	C	C	G	C	C	C	G	C	C
<i>S. wallichii</i> (72*)	AB206266	A	T	C	C	C	C	G	C	C	C	G	C	C	C	G	A	C
<i>S. juncoides</i> (74*)	AB206260	C	T	C	C	C	C	G	C	C	C	G	C	C	C	G	A	C
<i>S. lineolatus</i> (74*)	AB206262	C	C	T	T	C	C	G	T	T	C	A	T	C	T	A	C	C

		493	494	499	516	523	537	546	559	560	578	584	588	592	593	599	620
<i>S. komarovii</i> (38*)	AB206261	-	C	T	T	C	-	C	G	C	G	C	-	T	T	C	-
<i>S. hondoensis</i> (38*)	AB206258	-	C	T	T	C	-	C	G	C	G	T	-	T	T	C	-
<i>S. hotarui</i> (42*)	AB180720	-	C	T	T	C	-	C	G	C	G	T	-	T	T	C	-
<i>S. hotarui</i> (44*)	AB206259	-	C	T	T	C	-	C	G	C	G	T	-	T	T	C	-
<i>S. mucronatus</i> (38*)	AB206263	-	C	T	T	C	-	C	G	C	A	C	-	T	T	C	-
<i>S. triangulatus</i> (42)	AB206265	-	C	T	T	C	-	C	G	C	G	C	-	T	T	C	-
	AB523849	-	C	T	T	C	-	C	G	C	G	C	-	T	T	C	-
	AB523850	-	C	T	T	C	-	C	G	C	G	C	-	T	T	C	-
<i>S. ×trapezoideus</i> (43*)	AB523851	-	C	T	T	C	-	C	G	C	G	C/T	-	T	T	C	-
	AB523852	-	C	T	T	C	-	C	G	C	G	C/T	-	T	T	C	-
hybrid (60)	AB523853	-	C	T	T	C/T	-	C	G	C	G	C/T	-	T	T	C	-
<i>S. gemmifer</i> (76)	AB523848	-	C	T	T	T	-	C	G	C	G	C	-	T	T	C	-
	AB206257	-	C	T	T	T	-	C	G	C	G	C	-	T	T	C	-
<i>S. multisetus</i> (70*)	AB206264	-	C	T	T	C	-	C	G	C	G	C	-	T	T	C	-
<i>S. wallichii</i> (72*)	AB206266	A	T	T	T	C	C	C	T	T	G	T	A	C	A	C	A
<i>S. juncoides</i> (74*)	AB206260	-	C	T	A	C	-	C	T	T	G	T	-	T	A	C	-
<i>S. lineolatus</i> (74*)	AB206262	-	C	A	T	C	-	T	G	C	G	C	-	T	T	A	-

except for one at sequence position 200 (Table 3). An ITS sequence with the additive state of the parental sequences for hybrids species also has been reported in *Paeonia* L. (Paeoniaceae) (Sang *et al.* 1995) and  $\times$ *Crepidiastrixeris* Kitam. (Asteraceae) (Saito *et al.* 2006). They reported that this phenomenon in the nrITS sequences in hybrids might reflect their origin from different parental species. Our sequence results also suggested that the putative hybrid from Yawata ( $2n = 60$ ) arose by natural hybridization between *S. gemmifer* ( $2n = 76$ ) and *S. hotarui* ( $2n = 44$ ).

The ITS sequences of *Schoenoplectus hotarui* ( $2n = 44$ ) and *S. hondoensis* (Ohwi) Soják ( $2n = 38$ ) were almost the same. The combination of the ITS sequences of *S. hotarui* ( $2n = 44$ ) and *S. hondoensis* ( $2n = 38$ ) can explain all six additive sites of the putative hybrid perfectly. *Schoenoplectus hondoensis*, however, is mainly distributed in marshes in the high mountains of northern Honshu (Hayasaka 2002), while *S. gemmifer* is mainly distributed in wetlands and low mountains of western Japan (Kitamura *et al.* 2009). It is thought that *S. hondoensis* and *S. gemmifer* do not grow sympatrically. Thus, the putative hybrid in Yawata moor is believed to be a natural hybrid between *S. gemmifer* and *S. hotarui*, and not between *S. gemmifer* and *S. hondoensis*.

*Schoenoplectus*  $\times$ *trapezoideus* ( $2n = 43$ ) also showed additions, G/A or T/C, at the six sites (Table 3), indicating its hybridity. These additive sites can be explained by the combination of *S. hotarui* ( $2n = 44$ ) and *S. triangulatus* ( $2n = 42$ ), with one exception at sequence position 200, as in the new putative hybrid from Yawata. Although *S. hondoensis* has nearly the same ITS sequence as *S. hotarui*, the involvement of *S. hondoensis* in the origin of *S.*  $\times$ *trapezoideus* may be unlikely, since *S. hondoensis* has a chromosome number of  $2n = 38$  (Yano & Hoshino 2005). In this study, ITS data also supports the possibility that *S.*  $\times$ *trapezoideus* ( $2n = 43$ ) might have arisen by hybridization between *S. hotarui* ( $2n = 44$ ) and *S. triangulatus* ( $2n = 42$ ).

The ITS sequences data of putative hybrid from Yawata ( $2n = 60$ ) and *Schoenoplectus*  $\times$ *trapezoideus* ( $2n = 43$ ) showed an additive state

at six sites. However, sequence position 200 did not show an additive state, which was expected from the combinations of the putative parental species. These observations suggest that *S. hotarui* ( $2n = 44$ ) has nucleotide polymorphisms at this site. To clarify the origin of these hybrids without a doubt, additional ITS sequences of more individuals of *S. hotarui* ( $2n = 44$ ) need to be determined.

In *Schoenoplectus*, Greef & Triest (1999) suggested natural interspecific hybridization between *S. tabernaemontani* and *S. triqueter*, based on RAPD analysis. Fay *et al.* (2003) also suggested natural interspecific hybridization between *S. tabernaemontani* and *S. triqueter* using AFLP analysis. In our study, molecular and cytological combination data provided clear evidence for natural hybridization in *Schoenoplectus*. The ITS sequence data proved useful for demonstrating the origin of hybrids in Cyperaceae.

Our cytological and molecular data show the putative hybrid from Yawata moor to be a natural hybrid between *Schoenoplectus gemmifer* and *S. hotarui*, and not *S.*  $\times$ *trapezoideus*. *Schoenoplectus gemmifer* is a new species recently described from Kumamoto Prefecture by Sato *et al.* (2004). Natural hybrids between *S. gemmifer* and *S. hotarui* are thought to be derived from a new parental combination. Morphological features between the hybrids from Yawata moor and *S.*  $\times$ *trapezoideus* overlap. Further morphological analysis is needed before describing these plants as a new natural hybrid between *S. gemmifer* and *S. hotarui*.

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